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Potential Reservoirs and Risk Factors for VHSV IVb in an Enzootic System: Budd Lake, Michigan, United States

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Abstract.—Viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) has caused major, sporadic fish die-offs in the Laurentian Great Lakes region of North America since 2005. Presently, factors affecting VHSV IVb persistence in enzootic systems are not well understood. Even with annual surveillance, the virus can go undetected for several years following an outbreak before again re-emerging, which suggests the virus may be maintained in the system either below detectable levels or in un-tested reservoirs. The aim of this study was to identify potential reservoirs of VHSV IVb in Budd Lake, Michigan. VHSV IVb was first detected in Budd Lake in 2007, but then was undetected until 2011. Additionally, we explored the susceptibility of naïve fish introduced into a waterbody enzootic for VHSV IVb by stocking age-0 Largemouth Bass *Micropterus salmoides* at varying densities into enclosures in the lake. VHSV IVb was not detected among samples of *Notropis* spp. and *Lepomis* spp. fishes, Cylindrical Papershell mussels *Anodontoides ferussacianus*, leeches (subclass Hirudinea), sediment, or water. However, the virus was successfully isolated from amphipods (family Hyalellidae) and Largemouth Bass held in the enclosures. Our finding of VHSV IVb in Hyalellidae amphipods in combination with other research that has detected the virus in *Diporeia* spp., a large benthic amphipod important as a food resource to Great Lake fishes, suggests that benthic macroinvertebrates could be a reservoir for VHSV IVb in infected systems. If there are environmental reservoirs for VHSV IVb in infected systems, they are likely unevenly distributed. Findings of this study add to our understanding of the seemingly complex ecology of this deadly and economically detrimental virus.

Within the last decade, viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) has emerged in the Laurentian Great Lakes region of North America (Elsayed et al. 2006). The virus has been responsible for several mass mortality events, including those involving Freshwater Drum *Aplodinotus grunniens* and Round Goby *Neogobius melanostomus* in Lake Ontario in 2005 and 2006, and Gizzard Shad *Dorosoma cepedianum*, Yellow Perch *Perca flavescens*, and Muskellunge *Esox masquinongy* in Lake St. Clair in 2006 (Groocock et al. 2007; Lumsden et al. 2007; Faisal et al. 2012). In 2007, a Freshwater Drum die-off also occurred in Lake Winnebago, Wisconsin (Wisconsin Department of Natural Resources 2007). These mortality events and the spread of the virus into each of the Great Lakes as well as small waterbodies in the region prompted governmental agencies to implement a variety of control measures to limit its spread into federal and state fish hatcheries and other systems (Faisal et al. 2012). These control measures included implementation of strict biosecurity measures at culture facilities, thorough health examinations of broodstock at gamete collection facilities, and egg disinfection. Additional efforts to prevent the spread of the virus included implementation of baitfish health certification programs, prohibition of intra- and interstate movement of fishes, recommendations that anglers empty bilges and live wells and disinfect boats when leaving waterbodies, and expanded surveillance efforts (Faisal et al. 2012).

Considerable research has been conducted on VHSV IVb, unraveling important aspects of its molecular characteristics (Elsayed et al. 2006; Thompson et al. 2011; Pierce and Stepien 2012), hosts (Bain et al. 2010; Kim and Faisal 2010a; Goodwin and Merry 2011; Groocock et al. 2012), and geographical range (Faisal et al. 2012; Cornwell et al. 2015). Factors that affect its persistence in enzootic waterbodies have also been investigated (Hawley and Garver 2008; Cornwell et al. 2014). After a system has experienced a VHSV IVb outbreak, the virus

frequently goes undetected for a period of time before it again re-emerges. For example, in Lake St. Clair, VHSV IVb was identified in fish samples from 2003, 2006, and 2009, although the lake was inspected often (Faisal et al. 2012). Similarly, in Budd Lake, Michigan, VHSV IVb was detected in 2007 and 2011, but sampling conducted in intervening years failed to confirm the continuous presence of the virus (Faisal et al. 2012; Throckmorton et al. 2015, M. Faisal, unpublished data).

The inconsistent ability to detect VHSV IVb in enzootic waterbodies suggests the presence of environmental reservoirs that are either untested during surveillance efforts or that maintain the virus below the detection levels of currently approved diagnostic assays. Other factors may also contribute to latency and re-emergence of VHSV IVb. For example, a herd immunity response to the initial outbreak may lead to suppression of the virus for a period of time (Purcell et al. 2012, Standish 2016). Additionally, Kim and Faisal (2012) provided evidence that fish that survived VHSV IVb infection cease to shed the virus within a few weeks; however, shedding can resume during periods of stress. Kim and Faisal (2012) also suggested that stress-induced shedding could be a mechanism by which VHSV is maintained in an endemic waterbody. Fish possessing lower susceptibility to VHSV IVb, such as salmonids, may also help maintain the virus in endemic waterbodies as individuals may become infected with the virus but never succumb to disease (Kim and Faisal 2010a). Clearly, given the pathogenicity of the virus, a better understanding of the factors that may lead to its reemergence in previously-infected systems is highly desirable.

The natural reservoir host for VHSV IVb is susceptible fish, but like other rhabdoviruses, there may be less obvious incidental reservoir hosts that play a role in maintaining infectious virus particles within an ecosystem. For example, freshwater turtles have experimentally been

shown to be positive for VHSV IVb after consuming infected fish (Goodwin and Merry 2011). Additionally, amphipods of the genus *Diporeia* (Faisal and Winters 2011) and the *Myzobdella lugubris* leech (Faisal and Schulz 2009) have been found to harbor the virus. Knowing the full range of reservoir hosts for a particular pathogen is an important tool for pathogen control and would enhance both our understanding of VHSV IVb ecology and the effectiveness of managing against future harm stemming from the virus.

The primary goal of this study was to identify potential reservoirs of VHSV IVb in Budd Lake, Michigan, an inland lake in which VHSV IVb has been detected multiple times. Budd Lake was selected for this research because its history with the virus exemplifies the inconsistent ability to find VHSV IVb in an infected waterbody. The initial VHSV IVb outbreak in Budd Lake was first detected in May 2007 after a large-scale fish die-off occurred. Fish sampling conducted by the Michigan Department of Natural Resources and testing performed by the Michigan State University Aquatic Animal Health Laboratory confirmed VHSV IVb infections in Black Crappies *Pomoxis nigromaculatus*, Bluegills *Lepomis macrochirus*, Pumpkinseeds *L. gibbosus*, and Largemouth Bass *Micropterus salmoides (*Faisal et al. 2012). In contrast, one month after this initial detection, VHSV IVb could not be isolated from Bluegills, Pumpkinseeds, Largemouth Bass, Bluntnose Minnows *Pimephales notatus*, Sand Shiners *Notropis stramineus*, or Golden Shiners *Notemigonus crysoleucas* (Faisal et al. 2012). Fish samples collected by the Michigan Department of Natural Resources and tested by the Michigan State University Aquatic Animal Health Laboratory in 2008 and 2010 also failed to detect VHSV IVb (M. Faisal, unpublished data). In May 2011 (coincidentally on the same day that the enclosure study described below began), a mortality event occurred on Budd Lake involving Bluegill and Largemouth Bass, but VHSV IVb could not be isolated from any of the collected samples (M.

Faisal et al., unpublished data). However, shortly thereafter, active VHSV IVb infections were isolated in apparently healthy esocids (Muskellunge and Northern Pike *Esox lucius*) and Largemouth Bass at prevalences of 14% and 10%, respectively (Throckmorton et al. 2015).

An additional objective of this study was to assess if susceptible naïve fish can become infected when introduced into an enzootic VHSV IVb waterbody, without being allowed to be in physical contact with other fish residents of the lake. Answers to both questions addressed in this study will shed light on the ecology of this VHSV sublineage in an enclosed endemic waterbody.

<A>Methods

Study area.—Budd Lake (44°01′22″ N, 84°47′48″ W) is a 71-ha lake located in Clare County, Michigan. It is a morainic, calcareous seepage lake (Coffey and McNabb 1974) so there are no inlets or outlets, and the main water sources are precipitation and runoff supplemented by groundwater.

Fish collection and processing.—Fish from the *Lepomis* (Bluegills and Pumpkinseeds) and *Notropis* (Blackchin Shiner *N. heterodon*, Mimic Shiners *N. volucellus*, and Emerald Shiners *N. atherinoides*) genera were collected by pulsed-DC boat electrofishing (*Lepomis* spp.) or seining (*Notropis* spp.) in spring (April 22, May 2, May 9), summer (July 11, July 18), and fall (September 12, September 29) 2011. These sampling periods were selected primarily to determine whether virus prevalence varied seasonally; however, the timing of sampling was also influenced by availability of sampling equipment. Stratified random sampling was conducted by dividing the lake into northern and southern sections in which approximately equal numbers of individuals were randomly selected from both sections. This helped to ensure that samples were

144 distributed from throughout the lake. Electrofishing was conducted along the entire shoreline of
145 the lake, whereas seining was conducted in specific north and south regions that were conducive
146 to sampling (Figure 1). For each taxon (*Lepomis* spp. and *Notropis* spp.), a sample size of at least
147 60 individuals per season was attained as recommended by the Great Lakes Fishery Commission
148 Model Program for Fish Health Management in the Great Lakes to detect 5% prevalence with
149 95% confidence and presumed perfect test accuracy (Phillips et al. 2014). Fish were transported
150 to the Michigan State University Research Containment Facility and held live for up to one week
151 until tissue collection. Individuals from different locations in the lake were not always held
152 separately, thus our analyses did not distinguish between the two locations. Fish from separate
153 collection events were never held in the same tanks.

154 Fish were euthanized with an overdose of tricaine methanesulfonate (Finquel) (0.25 g/L
155 of water) buffered with sodium bicarbonate (0.5 g/L). Length and weight were recorded for all
156 *Lepomis* spp., and for the first five individuals of each species from each season of *Notropis* spp..
157 Kidney, spleen, and heart were pooled for each individual *Lepomis* spp. and for every five
158 individuals from *Notropis* spp. A separate set of autoclaved tools were used for each pool of
159 samples. Blood was collected from the caudal vein of *Lepomis* spp. when possible (41% of
160 individuals). Tissues were placed into 4-oz. bags and all tissue and blood samples were stored at
161 4°C until processing. For all virus detection processes, including tissue collection, aseptic
162 techniques were used and protocols were followed as detailed in AFS–FHS Bluebook (2005).

163 Within 12 h of tissue collection, samples were processed for cell culture. Tissue samples
164 were mixed 1:4 (w/v) with Earle's minimum essential medium (EMEM) and tryptose phosphate
165 broth supplemented with 1 M tris buffer (Trizma base and Trizma hydrochloride, Sigma),
166 gentamycin sulfate (100 mg/ml; Sigma), penicillin (100 IU/ml; Invitrogen), streptomycin (0.1

mg/ml; Invitrogen), and Fungizone (250 μg/mg; Fisher Scientific). Samples were homogenized for 4 min with a paddle blender (Stomacher 80 Biomaster) at full speed. Approximately 1 mL of the mixed product was then centrifuged for 30 min at 5,000 RPM, and supernatant was stored at 4°C until inoculation onto cells. Sera from *Lepomis* spp. were extracted by centrifugation of blood for 10 min at 5,000 RPM within 12 h of collection, followed by storage at -80°C until use. Prior to use, serum samples were diluted 1:10 in diluent.

Tissue homogenate supernatants and sera were inoculated onto epithelioma papulosum cyprini cells (Fijan et al. 1983), which were grown on flat-bottom 96-well plates in media containing EMEM, tryptose phosphate broth, heat-inactivated fetal bovine serum (Gemini), 200 *mM* L-glutamine (Invitrogen), 1-M tris buffer (Sigma), penicillin (100 IU/ml; Invitrogen), streptomycin (0.1 mg/ml; Invitrogen), gentamycin sulfate (100 mg/ml; Sigma), and Fungizone (250 μg/mg; Fisher-Scientific). Cells were propagated for 24 to 48 h to develop a confluent monolayer, after which six replicate wells were inoculated with 30 μl per well for each sample. At 14 d post-infection, samples were passed onto fresh cells for an additional 14 d, and the resulting cytopathic effect was recorded after the 28-d period. Samples were stored at -80°C until molecular assays.

The extraction of RNA from all cell culture supernatants was conducted with a viral RNA kit (QIAamp) following the manufacturer's instructions. One-step reverse transcription real-time PCR was completed with the Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems), following the manufacturer's protocol. The program was as follows: 1 cycle of reverse transcription at 50°C for 30 min, 1 cycle of activation and denaturation at 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 s, annealing and amplification at 60°C for 40 s, and polishing at 72°C for 20 s. This assay has been demonstrated to be very sensitive and valuable as

190 a tool for VHSV surveillance (Warg et al. 2014). For fish and all other samples, testing
191 methodology was consistent across all sampling periods.
192
193 *Macroinvertebrate collection and processing.*—Leeches (subclass Hirudinea) and amphipods
194 (families Hyalellidae and Gammaridae) were manually collected from leaf matter near the
195 northern (one site) and southern (one site) shores of the lake in the summer (July 19) and fall
196 (September 29) 2011 (Figure 1). Amphipods were also collected in spring 2012 (May 29, 30, and
197 31) from 20 sites around the perimeter of the lake based on the results of the 2011 sampling.
198 Individuals of the same collection site were grouped into pools of five and placed into 1.5-mL
199 microcentrifuge tubes. To reduce the possibility of contamination on the outside of the
200 organisms, individuals were surface-sterilized with 70% ethanol before placement into the tubes.
201 Despite this surface-sterilization, we acknowledge the possibility that virus particles may have
202 adhered to the outside of these organisms and that positive detection in invertebrates does not
203 necessarily mean infection. The weight of organisms in each tube was multiplied by four to
204 obtain volume of diluent to be added, followed by homogenization with a mortar and pestle.
205 Cylindrical Papershell mussels *Anodontoides ferussacianus* were manually collected from littoral
206 areas located near an island in the middle of the lake in the spring (May 31), summer (July 18),
207 and fall (September 12) 2011 (Figure 1). All internal viscera was collected and grouped into
208 pools of five. Tissue weight was multiplied by four to obtain volume of diluent to be added.
209 Samples were homogenized at full speed for 4 min with a Stomacher 80 Biomaster laboratory
210 paddle blender. Approximately 1 mL was centrifuged at 5,000 RPM for 30 min, inoculated onto
211 cells, and tested for VHSV IVb by real-time PCR as described above.
212

213 *Water and sediment collection and processing.*—Water and sediment samples were manually
214 collected from the lake in 2011. Samples were collected from 10 locations (five from the
215 northern half of the lake, five from the southern half) in the spring (April 22), summer (July 19),
216 and fall (September 29), yielding a total of 30 water samples and 30 sediment samples in 2011
217 (approximately 100 mL or 100 g per sample). The five samples from each region in each season
218 were pooled, yielding six pools of water and six pools of sediment. In spring 2012 (April 18 and
219 May 13), sampling of water and sediment was expanded. Samples were collected from 20
220 locations throughout the lake; 10 L of water were pumped from 3.0 m below the water surface,
221 and sediment was collected from the same site using an Ekman grab. The 2012 samples were
222 processed individually (i.e., samples were not pooled). Water samples were supplemented with
223 0.5% fetal bovine serum to aid in stabilization of the virus. After each water sample was
224 obtained, the pump and tubing were disinfected, dechlorinated, and rinsed. This was
225 accomplished by circulating 10% bleach, 10% sodium thiosulfate, and lake water through the
226 pump for 10 min each prior to collecting the next sample. Samples were stored at -20°C until
227 processing.

228 A tangential-flow filtration system was used to filter virus from water and sediment
229 samples with a 500-kDa hollow fiber filter (Spectrum Labs) after prefiltration through 20-µl and
230 2.5-µl filters. Tubing and filter were precooled by pumping cold, ultra-pure water through the
231 system. Pump speed was 200 mL/min, pressure was held between 1.5 and 2 psi, and the system
232 was back-flushed every 5 min or less. Retentate (approximately 120 mL) was further
233 concentrated via ultracentrifugation at 25,000 RPM for 2.5 h at 4°C, and the resulting pellet was
234 stored at -80°C until RNA extraction, reverse transcription, and real-time PCR as described
235 previously. The protocol for sediment was similar, except for the addition of chemical buffers to

disrupt the electrostatic and hydrophobic interactions between the virus and sediment particles (Gerba 1984). This addition to the protocol was adopted from Wommack et al. (2009). For each 100-g sediment sample, 400 mL of 10-mM sodium pyrophosphate and 40 µl of 5-mM EDTA was added. This mixture was placed onto a mixing plate for 20 min. After samples were gravity-filtered through cheesecloth followed by vacuum filtration through 20- and 2.4-µm filters, sediment filtrate underwent tangential-flow filtration and centrifugation as described for the water samples.

Exposure of naïve Largemouth Bass to the Budd Lake environment.—On May 3 2011, certified VHSV-free age-0 Largemouth Bass (Jones Fish Hatcheries, Newton, Ohio) were stocked into 54 enclosures distributed along northern and southern shores of the lake (Figure 1). Enclosures were stocked at one of three densities [5 (23.4 g mean biomass), 10 (43.9 g mean biomass), or 15 fish (65.0 g mean biomass)] per enclosure. In total, there were 18 enclosures per density. Enclosures were constructed of a PVC pipe frame encased within a vinyl-coated polyester mesh. The volume of each enclosure was approximately 1.7 m^3. Enclosures were arranged in blocks of three (one of each density per block). Enclosures were removed every four weeks throughout the summer: 15 (5 enclosures of each density) were removed on May 31 and June 28 and 12 (4 enclosures of each density) were removed on July 26 and August 23. Fish were brought back to the Michigan State University Research Containment Facility and held live until dissection. Tissues and sera were pooled into groups of five per pool, yielding a maximum of 1, 2, and 3 pools from the 5, 10, and 15 density enclosures, respectively. Due to mortalities, some pools contained less than five individuals and some pools contained one extra individual so that it was not in a separate pool on its own. Tissues and sera from live and moribund individuals at the time

of cage removal were collected and processed as described above for fish collected from Budd Lake. Because cage removal occurred every four weeks, testing dead fish for VSHV IVb was not possible and thus we cannot be certain whether mortalities resulted from VHSV IVb infection or some other cause, such as nutritional deficiency or thermal stress. Therefore, we did not evaluate mortalities in the cages as part of this study.

Data analysis—Prevalence of VHSV IVb for the different sample types was calculated using one of two approaches depending on whether samples were pooled or not. For individually-processed samples, (i.e., *Lepomis* spp. tissue/serum and 2012 water and sediment samples), prevalence was calculated by dividing the number of VHSV IVb-positive samples by the total number of samples. Confidence intervals (95%) for these rates were calculated using the complementary log parameterization in the binom package (Dorai-Raj 2014) in R (R Core Team 2014). For all other sample types in which individuals were pooled prior to testing, prevalence was calculated using the maximum likelihood approach described in Williams and Moffitt (2005). Confidence intervals (95%) for prevalences from pooled samples were calculated in R using the code provided in Williams and Moffitt (2005). For both cases, we implicitly assumed that the test for VSHV IVb was 100% specific and sensitive (Williams and Moffitt 2005), although we address this assumption later in the Discussion.

<A> Results

VHSV IVb was not isolated from any of the *Lepomis* or *Notropis* spp., nor was it isolated from mussels, leeches, water samples, or sediment samples (Table 1). Two of thirty-nine pools of amphipods from 2011 displayed amplification of VHSV IVb via real-time PCR for a total

prevalence of 1.0% (95% confidence interval: 0.2-3.2%). One pool was collected in the summer, and one in the fall. Both pools were collected from the northern end of the lake. Pooling of amphipod samples in 2011 was done irrespective of family so we cannot be certain whether the positive samples were Hyalellidae or Gammaridae. During the 2012 sampling, an additional 3 of 266 pools of amphipods tested positive for the virus for a total prevalence of 0.2% (95% confidence interval: 0.1-0.6%). The amphipods collected in 2012 that tested positive for VHSV IVb were identified as members of the family Hyalellidae. All pools were collected in the spring; two pools were collected from the northern end of the lake, and one in the southern end. Samples were only positive via real-time PCR; visible cytopathic effects were not evident with cell culture.

Naïve Largemouth Bass maintained in enclosures tested positive for VHSV IVb in two pools, one each of tissue and serum samples (Table 2). The positive tissue pool was from individuals in a high-density enclosure (15 fish) that was located near the southern end of the lake and was removed in June 2011. The positive serum pool was from individuals in a separate enclosure containing 10 fish that was located in the southern end of the lake and was removed in July 2011. As with the positive amphipod samples, all of the positive naïve Largemouth Bass samples were detected via real-time PCR, but the virus did not produce any visible cytopathic effects.

<A> Discussion

The positive virus detection in amphipods and stocked Largemouth Bass held in enclosures lends support to the contention that Budd Lake, Michigan, continues to be enzootic for VHSV IVb (Throckmorton et al. 2015). Throckmorton et al. (2015) isolated VHSV IVb

from Largemouth Bass and esocids in 2011, which coincided with our enclosure study and the first year of amphipod collection. However, it is of interest to note that Throckmorton et al. (2015) did not find any indication of virus presence in Largemouth Bass in spring 2012, even though in this study we detected VHSV IVb in amphipods during the same time period.

The taxonomic identification of the positive amphipod pools from 2011 is unknown; however, all positive samples in 2012 belonged to the Hyalellidae family. Hyalellidae amphipods are a food resource for species such as Largemouth Bass (Randall and Scalet 1975; Clady 1981), Bluegills (Crowder and Cooper 1982; Schramm and Jirka 1989) and Black Crappies (Schramm et al. 1985). Consequently, consumption of Hyalellidae amphipods could contribute to VHSV IVb persistence and infection in enzootic waterbodies, particularly given that juvenile fish have been found to be less resistant to infection compared with older individuals (Kocan et al. 1997; Kocan et al. 2001; Marty et al. 2003; Hershberger et al. 2007; Kim and Faisal 2010b). According to the research of Schönherz et al. (2012) on rainbow trout *Onchorynchus mykiss*, oral transmission of VHSV is possible. However, the progression of infection from this route of transmission is slower than compared with waterborne transmission. Schönherz et al. (2012) theorized that oral transmission of VHSV would be more important during initiation of infection in naïve populations, whereas waterborne transfer of the virus may be more substantial once an epizootic becomes established. For enzootic systems, either oral transmission stemming from consumption of infected individuals, including reintroduction by baitfish (Getchell et al. 2013) or waterborne transmission stemming from viral shedding by infected individuals (Kim and Faisal 2012), could play roles in periodic reemergence of the virus. Additional research clarifying the roles of these transmission routes in enzootic systems would be beneficial.

Our detection of VHSV IVb in amphipods in both 2011 and 2012 is consistent with the findings of Faisal and Winters (2011) who found that *Diporeia* spp. can test positive for the virus. *Diporeia* spp. are large benthic amphipods in the Great Lakes that have traditionally been an important food resource for numerous benthic species (Pothoven et al. 2001; Pothoven and Nalepa 2006). Thus, our results along with those of Faisal and Winters (2011) may be suggestive of a general role that macroinvertebrates play in VHSV IVb transmission and persistence. Whether or not oral transmission of VHSV IVb in enzootic systems occurs has not yet been determined, but a number of interesting questions stem from this possible transmission route and how it might be influenced by food resource dynamics of an aquatic community. In particular, the magnitude of this transmission route would depend on the susceptibility of a species. It would also depend on its realized niche stemming from availability of food and habitat and the presence of other predators and competitors. Therefore, the role that oral transmission of VHSV IVb plays in enzootic systems may be highly variable. Variability would depend on whether limiting factors result in the lack of contact between the virus and highly susceptible species or conversely result in high exposure to the virus.

Our failure to detect VHSV IVb in Bluegills and Pumpkinseeds (two species that are known to be susceptible to the virus) during a period of active infection in Budd Lake corroborates the inconsistencies in virus detection in virus-plagued water, and this is a hindrance that frustrates scientists and managers alike (Eckerlin et al. 2011; Faisal et al. 2012; Cornwell et al. 2014). These findings highlight the difficulty associated with obtaining a clear delineation of the spatial and temporal distribution of VHSV IVb in an infected waterbody. In particular, if VHSV IVb surveillance programs rely on opportunistic sampling or if they only sample one or

two components of an aquatic community, the surveillance may easily fail to detect the virus even though there are active infections in other members of the aquatic community.

Although VHSV IVb was not detected in any *Lepomis* or *Notropis* spp., leeches, or mussels, we caution that this does not entirely exclude the possibility that some of the individuals were harboring low amounts of virus that were below detection levels. In Atlantic Herring *Clupea harengus* populations in Scotland, individuals have tested positive for VHSV in the past but even sensitive real-time PCR assays have not been able to detect the virus in recent sampling events. This is despite the fact that the species is still believed to transfer the virus from wild to captive populations (Matejusova et al. 2010). Even with the most sensitive diagnostic assays such as the real-time PCR assay used in our study, false negative results can be obtained due to sampling site selection and the sampling time relative to disease course. It is also possible that other size- or age-classes of individuals were not vulnerable to our sampling gear (e.g., larval fish) and we simply failed to collect infected individuals. In addition, the strong susceptibility of larval fish can result in rapid mortality and thus make it difficult to capture infected individuals. One factor that may have contributed to the failure of virus detection from leeches is that the ones we collected in this study were not ectoparasites of fish (i.e., they were not from the family Piscicolidae), as they were in Faisal and Schulz (2009). Because of this, they may have had less exposure to the virus compared with other types of leeches. Yet, the amphipods that tested positive were likely exposed through free virus in the water column or through ingesting infected particles or sediment.

Part of our motivation for collecting and testing mussels in this study was that prior research has found that mussels can concentrate some viral pathogens (e.g., *Hepatovirus A* and *Norwalk virus*) through their filter feeding capacity (Li-ping et al. 2013; Pavoni et al. 2013;

372 Roldán et al. 2013). As viruses are dispersed throughout the water, they may be circulated into
373 the feeding pathway of mussels and eventually accumulate in their digestive systems, which can
374 then become a source for continued infections (Roldán et al. 2013). However, we did not identify
375 mussels as a source of VHSV IVb infection in Budd Lake. Faust et al. (2009) found that some
376 bivalves have the ability to not only remove the avian *Influenza A* virus from water, but to reduce
377 its infectivity as well. This could be what some species of mussels are able to do with VHSV
378 IVb, and this potential phenomenon deserves further investigation. Ultimately, the positive or
379 negative contribution of bivalves is dependent upon whether they maintain or diminish the
380 infection and development of the virus (Faust et al. 2009).

381 Despite the failure to detect VHSV IVb in the abiotic samples (water and sediment), we
382 believe it is still possible for VHSV IVb to be maintained in water and/or sediment for an
383 indeterminate amount of time and that this could contribute to the virus remaining in a system.
384 We may have failed to detect the virus as a consequence of small sample sizes or because our
385 sampling events did not coincide spatially and/or temporally with the virus. It has been
386 established that VHSV is stable in the environment for several days depending on the
387 temperature and virus strain (Hawley and Garver 2008), and this was corroborated for VHSV
388 IVb by Bain et al. (2010). Pham et al. (2012) concluded that there may be fomites for VHSV
389 IVb, which are inanimate objects that can retain and transfer infectious organisms. Nevertheless,
390 there are difficulties in isolating the virus from water or sediment as such due to its presumed
391 low concentration in the lake (i.e., virus-positive fish displaying little to no clinical signs and the
392 intermittent temporal and spatial detection of the virus). Improved success in isolation from these
393 abiotic media may rely on an optimized method of extraction.

Regarding our experiment involving naïve Largemouth Bass, the fact that both positive pools were from enclosures that were located at the south end of the lake demonstrates that virus is present in this area of the lake. Also, since the positive pools were from enclosures containing 10 fish (serum) and 15 fish (tissue), stress may have played a role in infections. It is interesting that virus in serum appeared later than virus in tissue, since the general progression of viral infection begins in the bloodstream followed by invasion to the tissues (Dua 2012). Early viremia in most fish rhabdoviruses is of transient nature followed by a period during which the virus cannot be found in either sera or tissues of infected fish (Kim and Faisal 2010b, 2012). As the infection advances, the virus is found in multiple tissues including blood. Perhaps the stressed fish in denser enclosures experienced a more accelerated disease course with a short initial viremia, while the less-dense enclosure fish ran a slower disease course. Indeed, we currently do not know how the naïve Largemouth Bass contracted the infection in the absence of contact with other resident fish, but water and feeding on infected amphipods are possible infection routes. Regarding the positive results in both Largemouth Bass and amphipods, it is acknowledged that since detection rates were low and results were not confirmed with cell culture, the possibility of false positives does exist.

We undertook this study to identify potential reservoirs to VHSV IVb in an enzootic system to help improve understanding of factors that contribute to periodic latency and re-emergence of the virus. While we were able to identify amphipods as a possible source for the virus, additional information on other factors that can affect VHSV IVb replication, transmission, virulence, and persistence are needed.

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592

593 **Figure Captions**

594 Figure 1. Budd Lake, Clare County, Michigan. Ovals indicate northern and southern shores of
595 the lake where, in 2011, amphipods, leeches, and *Notropis* spp. were collected and Largemouth
596 Bass enclosures were placed. The island is represented by the polygon in the center of the lake,
597 from where mussels were collected.